Received: 10 August 2017 | Revised: 13 September 2017 | Accepted: 24 September 2017

DOI: 10.1111/odi.12790

ORIGINAL ARTICLE

WILEY ORAL DISEASES

Muscle fiber diameter assessment in cleft lip using image processing

MFJ Khan[1] | J Little[2] | L Abelli[3] | PA Mossey[4] | L Autelitano[5] | TC Nag[6] | M Rubini[1]

[1]Department of Biomedical and Specialty Surgical Sciences, Section of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara, Ferrara, Italy

[2]School of Epidemiology and Public Health, University of Ottawa, Ottawa, ON, Canada

[3]Department of Life Sciences and Biotechnology, Section of Biology and Evolution, University of Ferrara, Ferrara, Italy

[4]Craniofacial Development at the World Health Organization–collaborating Centre for Oral and Craniofacial Research, Dental Hospital and School, University of Dundee, Dundee, Scotland

[5]Regional Centre for Orofacial Clefts and Craniofacial Anomalies, Department of Cranio-Maxillo-Facial Surgery, San Paolo Hospital, University of Milan, Milan, Italy

[6]Department of Anatomy, All India Institute of Medical Sciences, New Delhi, India

Correspondence
Mohammad Faisal Jamal Khan, Department of Biomedical and Specialty Surgical Sciences, Section of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara, Ferrara, Italy.
Email: khnmmm@unife.it

Objective: To pilot investigation of muscle fiber diameter (MFD) on medial and lateral sides of the cleft in 18 infants with cleft lip with or without cleft palate (CL/P) using image processing.

Material and Methods: Formalin-fixed paraffin-embedded (FFPE) tissue samples from the medial and lateral sides of the cleft were analyzed for MFD using an image-processing program (ImageJ). For within-case comparison, a paired Student's *t* test was performed. For comparisons between classes, an unpaired *t* test was used.

Results: Image processing enabled rapid measurement of MFD with majority of fibers showing diameter between 6 and 11 μm. There was no significant difference in mean MFD between the medial and lateral sides, or between CL and CLP. However, we found a significant difference on the medial side ($p = .032$) between males and females.

Conclusion: The image processing on FFPE tissues resulted in easy quantification of MFD with finding of a smaller MFD on the medial side in males suggesting possible differences in orbicularis oris (OO) muscle between the two sexes in CL that warrants replication using larger number of cases. Moreover, this finding can aid subclinical phenotyping and potentially in the restoration of the anatomy and function of the upper lip.

KEYWORDS
cleft lip, cleft lip and palate, FFPE tissue samples, image processing, muscle fiber diameter

1 | INTRODUCTION

Cleft lip with or without palate (CL/P) is the most common craniofacial congenital anomaly worldwide. Labial architecture is dramatically altered in CL/P cases, with slight differences among sexes and cleft subphenotypes (Carroll & Mossey, 2012). The severity of clefting differs between the sexes (Carroll & Mossey, 2012; Sivertsen et al., 2008).

Of the structures affected in cleft lip (CL), the orbicularis oris (OO) muscle is of particular significance. This muscle is among those that control facial expression, forming the sphincter of the oral cavity, and is intimately engaged in maintaining upper lip form and function. The OO muscle is composed of different types of muscle fibers (Schiaffino & Reggiani, 2011), which can be grouped into two broad types (Pette & Staron, 1990): type I (slow twitch), characterized by small fiber diameter, resistance to fatigue, and slow contraction, and type II (fast twitch) with larger diameter, low resistance to fatigue, and fast contraction rate. Hence, the functional distribution of these muscle fiber types may modulate the OO muscle leading to change in shape of the lips and is integral to the actions of feeding (Jacinto-Goncalves, Gaviao, Berzin, de Oliveira, & Semeguini, 2004; Tamura, Matsushita, Shinoda, & Yoshida, 1998) and speaking (Raphael, Borden, & Harris, 2007; Rastatter & Dejarnette, 1984; Rastatter, McGuire, & Blair, 1987; Regalo et al., 2005; Standring, 2004).

The predominant hypothesis regarding normal development of the upper lip muscle is that the migration of the mesenchymal cells across the fused prominences forms a dense and continuous band of mesenchymal cells that give rise to OO muscle. It is possible that the delayed or failed union of lip primordial prominences to the formation of continuous OO muscle prevents the correct orientation of the OO muscles. This delay in, or failure of, merging results in varying degree of damage and reduction in OO-MFD and function in patients with oral clefts (Lazzeri et al., 2008).

Patients with CL only or CL and palate (CLP) may require a number of interventions to enable rehabilitation to be as complete as possible, one of which is surgical reconstruction of the OO muscles (Cohen, 2004). During primary surgical repair of the lip, it is not possible to reconstruct the muscle anatomically and physiologically due to the creation of scar tissue, and contraction of this on healing results in unsightly bulging of the muscle on either side of the lip scar, depressions, and asymmetries that are further noticeable during animation, and give the lip an unnatural look (Cohen, 2004).

Although many histological studies have contributed to understanding of the development of CL, few studies have been carried out on the histological changes in the OO muscle at the edges of the CL (Raposio, Bado, Verrina, & Santi, 1998; Wijayaweera, Amaratunga, & Angunawela, 2000) or comparisons between the medial and lateral edges of CL (Wijayaweera et al., 2000).

Mounting evidence indicates that the structure of the upper lip is intimately associated with the OO muscle (Schiaffino & Reggiani, 2011) and demonstrates varying distribution of OO muscle fiber types along the upper lip (Dong & Zheng, 2015). Fara, 1968 and Gundlach & Pfeifer, 1979 showed increased disorganization of muscle fiber with the degree of clefting. Moreover, previous histological studies evaluating the OO muscle on medial and lateral edges of CL reported paucity and less disorganized muscles on the medial side with a greater disorganization on the lateral side (Wijayaweera et al., 2000).

Currently, ultrasound technology is used to visualize and assess the OO muscles in older children and adults (Weinberg et al., 2006). Using ultrasound to investigate OO muscle in first-degree relatives of probands with CL, three studies have found differences between OO muscles in first-degree relatives compared with controls having negative family history of CL/P in three generations and absence of any minimal cleft features (Martin et al., 2000; Mittal et al., 2012; Neiswanger et al., 2007). Neiswanger et al., 2007 also found differences in OO continuity between unaffected second- and third-degree relatives of CL/P probands and unrelated controls. These findings support the hypothesis of OO discontinuities as a subclinical phenotype in CL cases. However, there are no studies available on variation in OO-MFD (critical factors that determine the health and function of the muscle) on either side of the CL.

This evaluation of OO-MFD in infants with CL, in addition to the widely used histological and ultrasound approaches, might in future aid subclinical phenotyping and contribute to improve reconstructive approaches for CL. Therefore, in this pilot study, we sought to ascertain differences in muscle fiber diameter (MFD) on the medial and lateral sides of cleft lip by applying an image-processing tool (ImageJ) on Formalin-fixed paraffin-embedded (FFPE) OO muscle cross sections from CL and CLP individuals. To our knowledge, this approach has not previously been reported. Because of the known differences in severity of clefting and labial architecture by sex and cleft subtype, we applied image analysis to assess possible differences in the OO-MFD by sex and cleft subtype.

2 | MATERIALS AND METHODS

2.1 | Cases

Infants with non-syndromic CL/P were identified in the context of the ongoing PENTACLEFT project (http://www.unife.it/progetto/pentacleft). The project was approved by local IRB (prot. N.08-2011), and case enrollment was dependent on written informed consent from one or both parents. Consecutive families were invited to enroll in the study at the Regional Centre for Orofacial Clefts and Craniofacial Anomalies, San Paolo Hospital, Milan, Italy, at the time of the first surgical intervention on the index child. Infants with recognized syndromic clefts or the Pierre Robin sequence were excluded from the study. The PENTACLEFT project protocol includes the recruitment of non-syndromic CL/P cases, their parents and maternal grandparents, and the collection of genomic DNA from peripheral blood or buccal swab samples. Lip tissue samples were collected from non-syndromic CL/P cases at time of first surgery.

2.2 | Tissue samples

Eighteen cases, with an average age in months of 6.5 (CI 5.06-7.11) at the time of surgery, were recruited in this study: nine males (five CL and four CLP cases) and nine females (four CL and five CLP cases). For all cases, the lip tissue samples were taken from both medial and lateral side of CL and immediately fixed in 10% buffered formalin solution. Samples were then transferred to laboratory at University of Ferrara where they were processed for paraffin embedding. Histological sections (6 μm) were cut and stained with hematoxylin and eosin (H&E) in line with general practice in pathology laboratories.

2.3 | Image acquisition and processing

Orbicularis oris muscle fibers were observed under 40X objective lens and images captured using Nikon Eclipse E600 microscope coupled with DS-5 M video camera, software Lucia G 4.81, Laboratory Imaging Ltd., Praha, Hostiva. Images were taken at a resolution of 1,280 × 960 pixel and saved in tiff format. At least three 40X magnification fields were acquired with at least 65 MFD measurements for each specimen.

The H&E-stained images were processed using ImageJ (Ver. ImageJ 1.49 hr, Dresden, Germany), an open-source image-processing program. The original H&E image was first converted to a gray scale image (Figure 1b) by "8bit" ImageJ command (Ferreira & Rasband, 2012). Once converted, the gray scale image was segmented by the

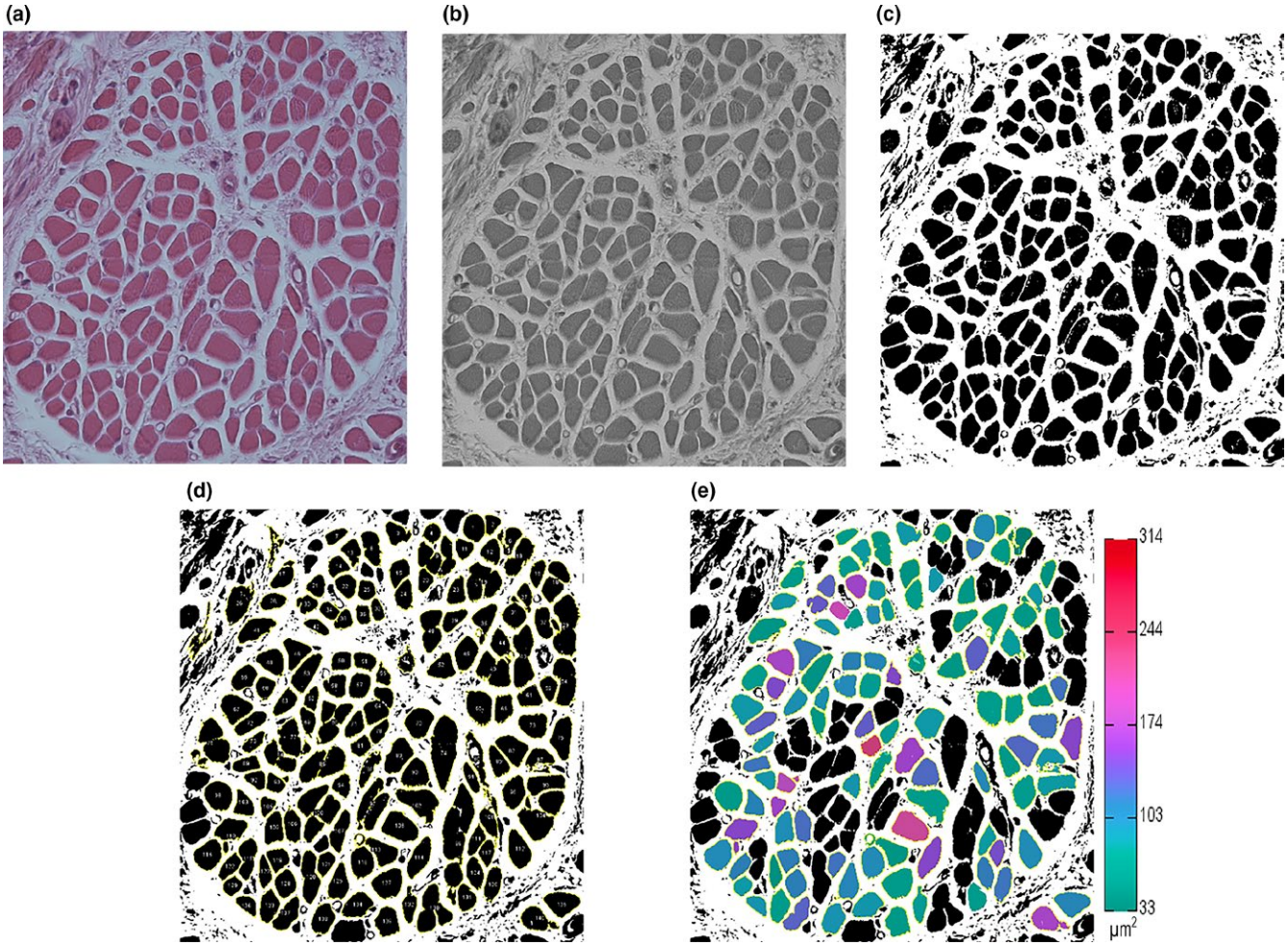

FIGURE 1 Preprocessing of micrographs using ImageJ standard commands to obtain digital color-coded image. (a) original H&E image; (b) 8-bit gray scale image; (c) thresholding image with "moments" scheme and "no dark background" converts image to binary format; (d) mask prepared by analyzing the particles with "no exclude edge" options. Output labeled muscle bundles; and (e) area-based color-coded muscle bundles for better visualization of sizes [Colour figure can be viewed at wileyonlinelibrary.com]

threshold command, using images comprising light objects on a dark background or dark objects on a light background. The lower and upper limits of the gray scale intensity were then adjusted so that the fibers of interest were clearly visualized. In general, a lower threshold limit of 0 and an upper limit of 130–150 for white backgrounds were found suitable for conversion of images to a binary format. The subsequent assigned commands in ImageJ were followed up for the input image to obtain the desired area of each OO muscle fiber. The sequence of events is illustrated in Figure 1.

As the internal calculations were performed in pixel units, it was necessary to convert these into physical units through calibration of the input image. Using the line tool of ImageJ, the number of pixels representing a known distance in the image was measured. These values, which change from image to image, were calculated for each input image to complete the calibration.

The "analyse particle" command was used to convert the preprocessed binary image (Figure 1d) into an estimated MFD area in physical units (μm^2). Color coding was prepared based on criteria that depended on standard output or calculated parameters (e.g., area). The color (Ferreira, 2014) overlaid on each muscle fiber (Figure 1e) helped in the visualization of different muscle fiber areas. The results were transferred to an Excel spreadsheet for further analyses.

2.4 | Statistical analysis

All the statistical analysis was performed using the IBM SPSS Statistics 21. MFD comparisons between medial and lateral side of cleft were carried out using paired Student's *t* test. The effect of sex or cleft type was assayed using unpaired Student's *t* test. The distributions of MFD were checked for normality using the Shapiro–Wilk test that is appropriate for small samples. Considering a number of 18 cases included in this study, at least 51 muscle fiber measurements per each tissue sample would be required to detect a 30% difference between cleft sides assuming an α-error (bilateral) of 0.05 and a β-error of 0.20. Similarly, the number of muscle fiber measurements per sample gave adequate statistical power to detect a 40% difference between sex groups or between cleft type groups.

3 | RESULTS

The processed image resulted in good color precipitation that covered the muscle fibers of interest with clear boundaries between muscle fibers (Figure 1a), and differences in staining intensities were easily adjustable using the threshold command with images comprising dark-colored MFDs on a white background (Figure 1c).

Our results are based on samples that showed normal distribution of MFD for both medial (p = .321) and lateral (p = .810) sides. The means and standard deviations of cleft tissue MFD between the medial and lateral sides, grouped according to sexes and cleft subtypes, are shown in Table 1. There was no significant difference (p = .095) of scores between the medial (9.09 ± 1.21 μm) and lateral (9.12 ± 1.82 μm) sides (Table 1).

The samples were also classified by sex of the infant, and compared for medial and lateral MFD. There was no significant difference between medial and lateral side within each sex. Comparison between sexes showed a nominally significant difference (p = .032) in score for the medial side, with males showing smaller diameter compared to females (Table 1).

Further analysis evaluated medial and lateral MFD by cleft subtype: CL and CLP. Comparison of medial and lateral MFD within CL (p = .378) and CLP (p = .427) showed no statistically significant differences. Likewise, comparison between CL and CLP showed no significant difference for medial side (p = .378) or lateral side (p = .379); Table 1.

In addition, we observed that the majority of estimated fiber diameters on both medial and lateral sides of cleft lip clustered in the range 6-11 μm (Figure 2).

4 | DISCUSSION

This pilot study demonstrates assessment of the OO-MFD in images of H&E stained samples obtained from FFPE lip tissues using image processing. We observed no significant difference in MFD between the medial and lateral sides of cleft lip. There was a significant difference between males and females in MFD for the medial side. There was no difference in MFD by cleft subtype.

On account of differences in analysis of OO muscle fibers in patients with cleft lip, the present study was designed to assess the OO-MFD by applying an image-processing method that to our knowledge has not been previously reported for cleft lip tissues.

Our imaging procedure demonstrated easy segmentation of the muscle fibers that capitalized on the measurement of MFD. We observed no significant difference (p = .957) in the MFD between the medial and lateral sides of the cleft. In medial tissue, males (8.49 ± 1.07 μm) had a smaller mean MFD than females (9.69 ± 1.09 μm), but based on the limited number of cases included in this preliminary study, the evidence can be considered as a suggestion that there is a difference. No significant differences were observed comparing medial and lateral sides within and between two cleft subtypes. Moreover, the majority of fiber diameters clustered within a range of 6-11 μm on both cleft sides (Figure 2). We acknowledge that our results are based on small numbers, because collecting tissues from the cleft cases is a challenge (Stock et al., 2016). We urge replication of our results in a larger sample size in future work.

The observed sex-based difference on the medial MFD could be a consequence of inherent sex-related developmental differences (Natsume et al., 1988), or differential response to hormones. For example, until the 11th week of gestation, progesterone production is governed by the maternal corpus luteum, after which its production is taken over by the fetal-guided placenta (Larsen, 2001). This shift from maternal to fetal hormone production occurs around the same time that the OO muscle appears (Neiswanger et al., 2007). If this shift differed by the sex of the developing fetus, it could result in a slight different effect on the formation of OO muscle fiber reflected as possible change in fiber diameter, as we observed.

TABLE 1 Orbicularis oris muscle fiber diameter (μm) of medial and lateral cleft sides. Mean±standard deviation (*SD*) values of total CL/P cases or cases categorized by sex or cleft subtype are shown, along with mean difference, 95% confidence interval (C.I.), and nominal p-value of t test

	Medial side±*SD*	Lateral side±*SD*	Mean difference (95% C.I.) p-value
Total cases (N = 18)	**9.09** ± 1.21	**9.12** ± 1.82	−0.02 (−1.16 to 1.10) p = .95
Males (N = 9)	**8.49** ± 1.07	**8.98** ± 2.30	−0.49 (−2.63 to 1.65) p = .61
Females (N = 9)	**9.69** ± 1.09	**9.25** ± 1.33	0.43 (−0.83 to 1.70) p = .45
Mean difference (95% C.I.) p-value	−1.19 (−2.28 to −0.11) **p = .03**	−0.26 (−2.14 to 1.61) p = .76	
CL (N = 9)	**9.35** ± 1.38	**8.72** ± 1.84	0.62 (−0.91 to 2.16) p = .37
CLP (N = 9)	**8.82** ± 1.03	**9.51** ± 1.83	−0.68 (−2.56 to 1.19) p = .42
Mean difference (95% C.I.) p-value	0.52 (−0.70 to 1.74) p = .37	−0.78 (−2.62 to 1.05) p = .37	

SD, standard deviation; CI, confidence interval; CL, cleft lip; CLP, cleft lip and palate.
Rows are showing paired comparison, and columns are showing unpaired comparisons. Mean and significant p-value are present in bold.

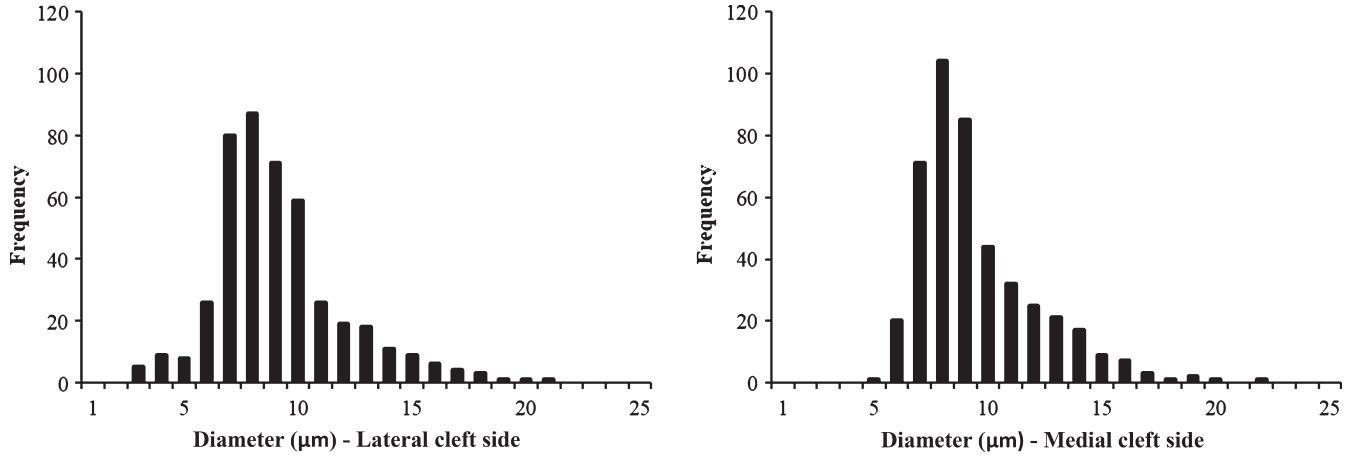

FIGURE 2 Frequency of muscle fiber diameter for medial and lateral cleft sides

Another possible reason could be shortage of early mesenchymal cells on the medial side of the lip that eventually form the continuous band corresponding to the future OO muscle (Lazzeri et al., 2008). Such a shortage of medial mesenchymal cells in CL has been reported in a recent study (Everson et al., 2017). We postulate that the proliferation and migration of the mesenchymal cells on the medial side may be different for the two sexes, leading to formation of non-continuous bands of OO muscles observed in the CL cases (Neiswanger et al., 2007), that possibly appear as differences in MFD as observed in our study.

The image processing employed in the present study on FFPE lip tissues resulted in proper segmentation of proximal fibers and automated the measurement of MFD. This may in future assist in overcoming the difficulties of the conventional manual procedure based on enzyme histochemistry (the ATPase method) that is critically sensitive to pH, temperature, time of incubation and requires frozen tissue specimens. In addition, our method can be readily employed within and as an alternative to routine laboratory assessment that enables use of FFPE tissues in OO-MFD measurement.

Moreover, we realize that our study also has some potential limitations with respect to recognition of muscle fiber type and validation of our technique in doing so. These limitations are imposed by lack of commercially available functional antibodies for FFPE tissues (Suriyonplengsaeng et al., 2017), and the possibility of overfixation that might mask the epitope (Ramos-Vara, 2005) or result in strong non-specific background staining, impeding validation by immunohistochemistry (IHC). However, a recent study has demonstrated that the use of commercially available antibodies, designed for use in fresh tissues, gives satisfactory results when used in the analysis of FFPE lip tissue (Dong & Zheng, 2015). For this reason, in future work we will validate our preliminary findings using images obtained by IHC.

Of note, our study is a pilot study with small sample size (with possibility of both type I and type II errors), and being aware of this limitation, splitting our samples based on factors (sex and cleft subtype) thought to affect muscle diameter was intended to provide preliminary data. We did not correct for multiple testing in view of the limitations of Bonferroni and other forms of adjustment (Armstrong, 2014; Perneger, 1998). As the recruitment of cleft cases is still ongoing in the PENTACLEFT project, we hope to replicate and justify our preliminary finding in new samples using immunohistochemistry coupled with our imaging procedure in a large number of cleft cases.

In conclusion, the image processing on FFPE tissues resulted in easy quantification of MFD that can serve as an alternative to, and gives results compatible with, established methods of assessing OO-MFD. We found that MFD on the medial side was smaller in male than in female infants. This could reflect the play of chance or possibly be a result of developmental differences in OO muscles between the two sexes. Moreover, our finding can in future aid subclinical phenotyping and restoration of the anatomy and function of the upper lip. This finding adds to evidence on the role of sex in the etiology of CL and warrants replication using a larger number of cases.

ACKNOWLEDGEMENTS

We acknowledge the support received from the European Science Foundation within the "Network for Orofacial Clefts Research, Prevention and Treatment" (EUROCleftNet, 09-RNP-023) program (MFJK, ESF exchange visit grants, n. 5023, 5152). Furthermore, our sincere thanks to Dr. Houda Oudouche, Department of Humanities, University of Ferrara, Italy and Lab. Group members: Rita Bassi Andreasi, Valentina Aleotti, Ilenia Lombardo, Martina Bonsi, Ilaria Cestonaro, and Luca Dall'Olio for their assistance. JL holds a tier 1 Canada Research Chair.

CONFLICT OF INTERESTS

None to declare.

AUTHOR CONTRIBUTIONS

MFJK designed the study, carried out experiment, and drafted the manuscript. MR and LA managed recruitment of PENTACLEFT lip tissue samples. The data were analyzed by MFJK together with MR. MR, JL, PM, and TCN checked and revised the manuscript. LA helped to perform experiment. All authors read and approved the final manuscript.

ORCID

MFJ Khan http://orcid.org/0000-0003-4365-2749
M Rubini http://orcid.org/0000-0003-1448-9516

REFERENCES

Armstrong, R. A. (2014). When to use the Bonferroni correction. *Ophthalmic and Physiological Optics*, *34*(5), 502–508. https://doi.org/10.1111/opo.12131

Carroll, K., & Mossey, P. A. (2012). Anatomical variations in clefts of the lip with or without cleft palate. *Plast Surg Int*, 2012, 542078. https://doi.org/10.1155/2012/542078

Cohen, M. (2004). Residual deformities after repair of clefts of the lip and palate. *Clinics in Plastic Surgery*, *31*(2), 331–345. https://doi.org/10.1016/S0094-1298(03)00133-0

Dong, C., & Zheng, S. (2015). Immunohistochemical analysis of orbicularis oris muscle fiber distribution at the philtrum in healthy infants. *International Journal of Pediatric Otorhinolaryngology*, *79*(12), 2208–2212. https://doi.org/10.1016/j.ijporl.2015.10.005

Everson, J. L., Fink, D. M., Yoon, J. W., Leslie, E. J., Kietzman, H. W., Ansen-Wilson, L. J., & Lipinski, R. J. (2017). Sonic Hedgehog regulation of Foxf2 promotes cranial neural crest mesenchyme proliferation and is disrupted in cleft lip morphogenesis. *Development*, *144*(11), 2082–2091. https://doi.org/10.1242/dev.149930

Fara, M. (1968). Anatomy and arteriography of cleft lips in stillborn children. *Plastic and Reconstructive Surgery*, *42*(1), 29–36.

Ferreira, T. (2014). *ROI Color Coder*. Ver. 5.1. ImageJ Documentation Wiki. http://imagejdocu.tudor.luFreund

Ferreira, T., & Rasband, W. (2012). ImageJ user guide, IJ 1.46r. https://imagej.nih.gov/ij/docs/guide/user-guide.pdf

Gundlach, K. K., & Pfeifer, G. (1979). The arrangement of muscle fibres in cleft lips. *Journal of Maxillofacial Surgery*, *7*(2), 109–116. https://doi.org/10.1016/S0301-0503(79)80022-3

Jacinto-Goncalves, S. R., Gaviao, M. B., Berzin, F., de Oliveira, A. S., & Semeguini, T. A. (2004). Electromyographic activity of perioral muscle in breastfed and non-breastfed children. *Journal of Clinical Pediatric Dentistry*, *29*(1), 57–62.

Larsen, W. J. (2001). *Human embryology* (3rd edn, pp. 544). New York: Churchill Livingstone.

Lazzeri, D., Viacava, P., Pollina, L. E., Sansevero, S., Lorenzetti, F., Balmelli, B., & Massei, A. (2008). Dystrophic-like alterations characterize orbicularis oris and palatopharyngeal muscles in patients affected by cleft lip and palate. *Cleft Palate-Craniofacial Journal*, *45*(6), 587–591. https://doi.org/10.1597/07-026.1

Martin, R. A., Hunter, V., Neufeld-Kaiser, W., Flodman, P., Spence, M. A., Furnas, D., & Martin, K. A. (2000). Ultrasonographic detection of orbicularis oris defects in first degree relatives of isolated cleft lip patients. *American Journal of Medical Genetics*, *90*(2), 155–161.

Mittal, M., Maheshwari, N., Ahlawat, K., Sharma, V., Sultan, A., & Chopra, R. (2012). Detection of orbicularis oris muscle defects in first degree relatives of cleft lip children using ultrasound. *Journal of the Indian Society of Pedodontics and Preventive Dentistry*, *30*(3), 237–241. https://doi.org/10.4103/0970-4388.105017

Natsume, N., Kaetsu, A., Kanoh, Y., Fukano, H., Makino, S., & Kawai, T. (1988). Regularity in the development of cleft lip and/or palate patterns. *Aichi-Gakuin Dental Science*, *1*, 57–64.

Neiswanger, K., Weinberg, S. M., Rogers, C. R., Brandon, C. A., Cooper, M. E., Bardi, K. M., & Marazita, M. L. (2007). Orbicularis oris muscle defects as an expanded phenotypic feature in nonsyndromic cleft lip with or without cleft palate. *American Journal of Medical Genetics. Part A*, *143A*(11), 1143–1149. https://doi.org/10.1002/ajmg.a.31760

Perneger, T. V. (1998). What's wrong with Bonferroni adjustments. *BMJ*, *316*(7139), 1236–1238.

Pette, D., & Staron, R. S. (1990). Cellular and molecular diversities of mammalian skeletal muscle fibers. *Reviews of Physiology Biochemistry and Pharmacology*, *116*, 1–76.

Ramos-Vara, J. A. (2005). Technical aspects of immunohistochemistry. *Veterinary Pathology*, *42*(4), 405–426. https://doi.org/10.1354/vp.42-4-405

Raphael, L. J., Borden, G. J., & Harris, K. S. (2007). *Speech science primer: Physiology, acoustics, and perception of speech* (5th edn). Philadelphia: Lippincott, Williams & Wilkins.

Raposio, E., Bado, M., Verrina, G., & Santi, P. (1998). Mitochondrial activity of orbicularis oris muscle in unilateral cleft lip patients. *Plastic and Reconstructive Surgery*, *102*(4), 968–971.

Rastatter, M., & Dejarnette, G. (1984). EMG activity with the jaw fixed of orbicularis oris superior, orbicularis oris inferior and masseter muscles of articulatory disordered children. *Perceptual and Motor Skills*, *58*(1), 286. https://doi.org/10.2466/pms.1984.58.1.286

Rastatter, M., McGuire, R., & Blair, B. (1987). EMG activity with the jaw fixed of orbicularis oris superior, orbicularis oris inferior and masseter muscles of mild and moderate articulatory disordered children. *Perceptual and Motor Skills*, *64*(3 Pt 2), 1213–1214. https://doi.org/10.2466/pms.1987.64.3c.1213

Regalo, S. C., Vitti, M., Moraes, M. T., Semprini, M., de Felicio, C. M., de Mattos Mda, G., & Santos, C. M. (2005). Electromyographic analysis of the orbicularis oris muscle in oralized deaf individuals. *Brazilian Dental Journal*, *16*(3), 237–242. https://doi.org/S0103-64402005000300012

Schiaffino, S., & Reggiani, C. (2011). Fiber types in mammalian skeletal muscles. *Physiological Reviews*, *91*(4), 1447–1531. https://doi.org/10.1152/physrev.00031.2010

Sivertsen, A., Wilcox, A., Johnson, G. E., Abyholm, F., Vindenes, H. A., & Lie, R. T. (2008). Prevalence of major anatomic variations in oral clefts. *Plastic and Reconstructive Surgery*, *121*(2), 587–595. https://doi.org/10.1097/01.prs.0000297839.78179.f5

Standring, S. (2004). *Gray's anatomy: The anatomical basis of clinical practice* (39th edn). London: Churchill Livingstone.

Stock, N. M., Humphries, K., Pourcain, B. S., Bailey, M., Persson, M., Ho, K. M., & Sandy, J. (2016). Opportunities and challenges in establishing a cohort study: An example from cleft lip/palate research in the United Kingdom. *Cleft Palate-Craniofacial Journal*, *53*(3), 317–325. https://doi.org/10.1597/14-306

Suriyonplengsaeng, C., Dejthevaporn, C., Khongkhatithum, C., Sanpapant, S., Tubthong, N., Pinpradap, K., & Waisayarat, J. (2017). Immunohistochemistry of sarcolemmal membrane-associated proteins in formalin-fixed and paraffin-embedded skeletal muscle tissue: A promising tool for the diagnostic evaluation of common muscular dystrophies. *Diagnostic Pathology*, *12*(1), 19. https://doi.org/10.1186/s13000-017-0610-y

Tamura, Y., Matsushita, S., Shinoda, K., & Yoshida, S. (1998). Development of perioral muscle activity during suckling in infants: A cross-sectional and follow-up study. *Developmental Medicine and Child Neurology*, *40*(5), 344–348.

Weinberg, S. M., Neiswanger, K., Martin, R. A., Mooney, M. P., Kane, A. A., Wenger, S. L., & Marazita, M. L. (2006). The pittsburgh oral-facial cleft study: Expanding the cleft phenotype. background and justification. *Cleft Palate-Craniofacial Journal*, *43*(1), 7–20. https://doi.org/10.1597/04-122R1.1

Wijayaweera, C. J., Amaratunga, N. A., & Angunawela, P. (2000). Arrangement of the orbicularis oris muscle in different types of cleft lips. *Journal of Craniofacial Surgery*, *11*(3), 232–235.

How to cite this article: Khan MFJ, Little J, Abelli L, et al. Muscle fiber diameter assessment in cleft lip using image processing. *Oral Dis*. 2018;24:476–481. https://doi.org/10.1111/odi.12790